

Preparing Samples for Small RNA Sequencing Using the Alternative v1.5 Protocol

FOR RESEARCH ONLY

Topics

- 3 Protocol Overview
- 3 Introduction
- 5 RNA Input Recommendations
- 6 Sample Preparation Kit Contents
- 8 User-Supplied Consumables and Equipment
- 9 Dilute Oligos and MgCl₂ for v1.5 Protocol
- 10 Ligate the v1.5 Small RNA 3' Adapter and 5' Adapter
- 11 Reverse Transcribe and Amplify the Small RNA Ligated with Adapters
- 13 Purify the Amplified cDNA Construct
- 19 Validate the Library

This document and its contents are proprietary to Illumina, Inc. and its affiliates ("Illumina"), and are intended solely for the contractual use of its customers and for no other purpose than to use the product described herein. This document and its contents shall not be used or distributed for any other purpose and/or otherwise communicated, disclosed, or reproduced in any way whatsoever without the prior written consent of Illumina, Inc.

For the proper use of this product and/or all parts thereof, the instructions in this document must be strictly and explicitly followed by experienced personnel. All of the contents of this document must be fully read and understood prior to using the product or any of the parts thereof.

FAILURE TO COMPLETELY READ AND FULLY UNDERSTAND AND FOLLOW ALL OF THE CONTENTS OF THIS DOCUMENT PRIOR TO USING THIS PRODUCT, OR PARTS THEREOF, MAY RESULT IN DAMAGE TO THE PRODUCT, OR PARTS THEREOF, AND INJURY TO ANY PERSONS USING THE SAME.

RESTRICTIONS AND LIMITATION OF LIABILITY

This document is provided "as is," and Illumina assumes no responsibility for any typographical, technical or other inaccuracies in this document. Illumina reserves the right to periodically change information that is contained in this document and to make changes to the products, processes, or parts thereof described herein without notice.

Illumina does not assume any liability arising out of the application or the use of any products, component parts, or software described herein. Illumina does not convey any license under its patent, trademark, copyright, or common-law rights nor the similar rights of others. Illumina further reserves the right to make any changes in any processes, products, or parts thereof, described herein without notice. While every effort has been made to make this document as complete and accurate as possible as of the publication date, no warranty of fitness is implied, nor does Illumina accept any liability for damages resulting from the information contained in this document.

ILLUMINA MAKES NO REPRESENTATIONS, WARRANTIES, CONDITIONS OR COVENANTS, EITHER EXPRESS OR IMPLIED (INCLUDING WITHOUT LIMITATION ANY EXPRESS OR IMPLIED WARRANTIES OR CONDITIONS OF FITNESS FOR A PARTICULAR PURPOSE, NON-INFRINGEMENT, MERCHANTABILITY, DURABILITY, TITLE, OR RELATED TO THE PERFORMANCE OR NONPERFORMANCE OF ANY PRODUCT REFERENCED HEREIN OR PERFORMANCE OF ANY SERVICES REFERENCED HEREIN).

This document may contain references to third-party sources of information, hardware or software, products or services and/or third-party web sites (collectively the "Third-Party Information"). Illumina does not control and is not responsible for any Third-Party Information, including, without limitation, the content, accuracy, copyright compliance, compatibility, performance, trustworthiness, legality, decency, links, or any other aspect of Third-Party Information. Reference to or inclusion of Third-Party Information in this document does not imply endorsement by Illumina of the Third-Party Information or of the third-party in any way.

© 2009 Illumina, Inc. All rights reserved.

Illumina, Solexa, Making Sense Out of Life, Oligator, Sentrix, GoldenGate, DASL, BeadArray, Array of Arrays, Infinium, BeadXpress, VeraCode, IntelliHyb, iSelect, CSPro, and GenomeStudio are registered trademarks or trademarks of Illumina, Inc. All other brands and names contained herein are the property of their respective owners.

Protocol Overview

This protocol explains how to prepare small RNA libraries for subsequent sequencing on the Illumina Cluster Station and Genome Analyzer.

This guide details the Small RNA Sample Preparation Alternative v1.5 Protocol. Libraries prepared by this method should be loaded only on single-read flowcells for cluster generation. The small RNA sequencing primer (part # 1001375) and single-read sequencing methods should be used for the sequencing reactions.

This protocol requires one of the following Illumina products:

- ▶ Small RNA Sample Prep Kit (8 samples) FC-102-1009
- ▶ Small RNA Sample Prep Kit (40 samples) FC-102-1010

Introduction

This protocol is designed to use either total RNA or purified small RNAs as input. You will ligate the adapters necessary for use during cluster creation, reverse-transcribe, and PCR amplify to generate the following template:

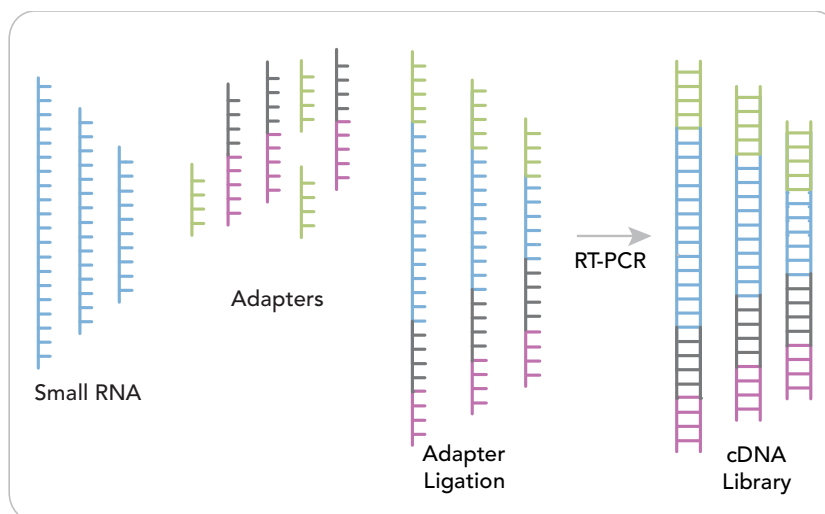


Figure 1 Fragments after Sample Preparation

The v1.5 small RNA 3' adapter is specifically modified to target microRNAs and other small RNAs that have a 3' hydroxyl group resulting from enzymatic cleavage by Dicer or other RNA processing enzymes. The 3' adapter is required for reverse transcription and corresponds to the surface-bound amplification primer on the flow cell. The 5' small RNA adapter is necessary for amplification of the small RNA fragments.

Sample Prep Workflow

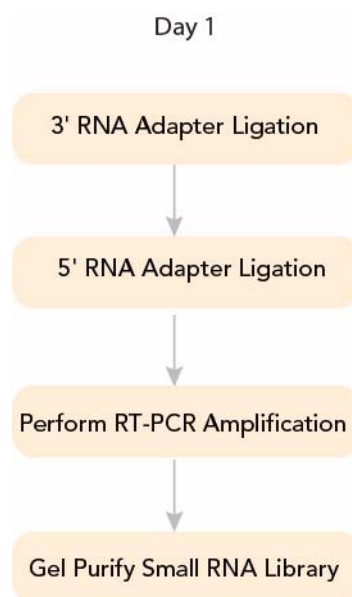


Figure 2 Sample Preparation Workflow



NOTE

The sample may be stored after ethanol precipitation, if necessary.

RNA Input Recommendations

RNA Input Recommendations

This protocol has been optimized using 1 µg of high-quality human or mouse brain total RNA as input. Use of RNA from other species, tissues, or qualities may require further optimization with regard to the initial input amount and selection of the desired bands during the final gel excision. The type and coverage of small RNAs sequenced will also vary depending on which bands are selected during gel excision.

Total RNA Input

To use total RNA as input, add 1 µg–10 µg of total RNA to the first ligation reaction. You will select the adapter-ligated small RNAs for sequencing during the subsequent gel extraction step. See the sample gels in Figure 5 for examples of how to select bands containing adapter-ligated small RNAs.

Purified Small RNA Input

To use previously isolated small RNA as input, add the entire fraction of small RNA purified from 1 µg–10 µg total RNA to the first ligation reaction. Fewer undesired bands will be seen during the subsequent gel extraction using this method. See the sample gel in Figure 6.

Sample Preparation Kit Contents

Check to ensure that you have all of the reagents identified in this section before proceeding to sample preparation.

Small RNA Sample Prep Kit, Box 1

Store at -15° to -25°C

This box is shipped at -80°C. As soon as you receive it, store the following components at -15° to -25°C.

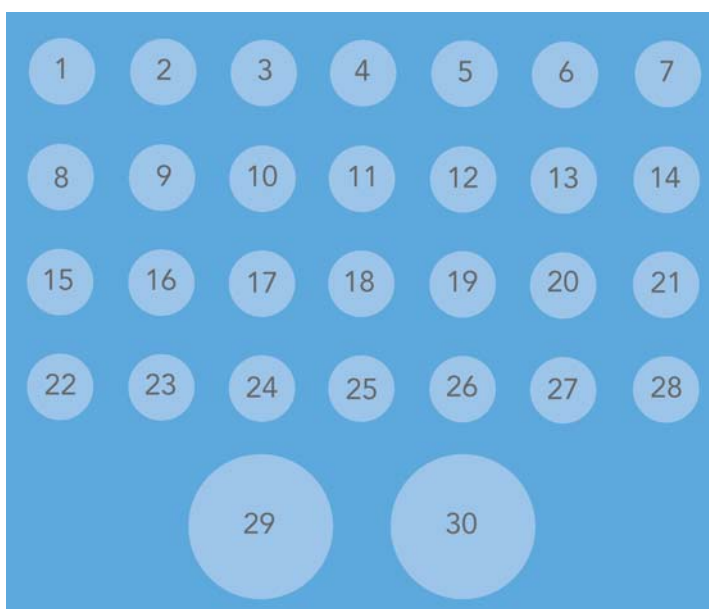


Figure 3 Small RNA Sample Prep Kit, Box 1

1. SRA Ladder, part # 1001665
2. SRA Gel Loading Dye, part # 1001661
3. Glycogen, part # 1001664
4. T4 RNA Ligase, part # 1000587
5. 10X T4 RNA Ligase Buffer, part # 1000588
6. RNase OUT, part # 1000560
7. SRA RT Primer, part # 1000597
8. 25 mM dNTP Mix, part # 1001663
9. Phusion Polymerase (Finnzymes Oy), part # 1000584
10. 5X Phusion HF Buffer (Finnzymes Oy), part # 1000585
11. Primer GX1, part # 1000591
12. Primer GX2, part # 1000592
13. 25 bp Ladder, part # 1001662

- 14. 10X Gel Elution Buffer, part # 1000571
- 15. Resuspension Buffer, part # 1001388
- 16. –28. Empty
- 29. Ultra Pure Water (store at 4°C), part # 1000467
- 30. SRA 0.3 M NaCl, part # 1000573

Small RNA Sample Prep Kit, Box 2

Store at Room Temperature

These components are shipped at room temperature.

- ▶ Spin X Cellulose Acetate Filter

Small RNA Sample Prep Kit, Bag 1

Store at -15° to -25°C

This bag is shipped at -80°C. As soon as you receive it, store the following components at -15° to -25°C.

- ▶ SRA 5' Adapter, part # 1000595
- ▶ SRA 3' Adapter, part # 1000596 (The PR 3' adapter is not used in the v1.5 protocol.)



NOTE

Briefly centrifuge the tubes before use, as the contents may have settled on the sides.

Small RNA Sample Prep v1.5 Conversion Kit, Bag 2

Store at -15° to -25°C

This bag is shipped at -80°C. As soon as you receive it, store the following component at -15° to -25°C.

- ▶ 10X v1.5 sRNA 3' Adapter, part # 15000263

All reagents are supplied in excess to guarantee you have the quantity necessary to perform eight small RNA sample preparations. It is normal to have leftover reagents following the preparation of eight samples.

User-Supplied Consumables and Equipment

Consumables

Check to ensure that you have all of the necessary user-supplied consumables before proceeding to sample preparation.

- ▶ 10 mM ATP (Epicenter, part # R109AT or any molecular grade substitute)
- ▶ T4 RNA Ligase 2, truncated with 10X T4 RNL2 truncated reaction buffer (NEB, part # M0242S)
- ▶ 10X T4 RNL2 truncated reaction buffer (NEB-supplied)
- ▶ 100 mM MgCl_2 (a 100 mM solution can be prepared from 1 M MgCl_2 (USB, part # 78641) or any molecular grade substitute)
- ▶ Ultra Pure Ethidium Bromide 10 mg/ml
- ▶ Room temperature 100% ethanol
- ▶ Room temperature 75% ethanol
- ▶ Room temperature 70% ethanol
- ▶ Clean scalpels
- ▶ 21-gauge needles
- ▶ 5X Novex TBE buffer
- ▶ SuperScript II Reverse Transcriptase with 100 mM DTT and 5X First Strand Buffer (Invitrogen, part # 18064-014)
- ▶ 6% Novex TBE PAGE gel, 1.0 mm, 10 well
- ▶ 3 M NaOAc, pH 5.2
- ▶ -20°C 100% ethanol
- ▶ 6X DNA loading dye
- ▶ Nuclease-free water
- ▶ 5' and 3' adapter-ligated RNA (4.0 μl)
- ▶ Clean 0.2 mL, 0.5 mL, and 2.0 mL nuclease-free microcentrifuge tubes
- ▶ 100 mM MgCl_2
(A 100 mM solution can be prepared from 1 M MgCl_2 (USB, part # 78641) or any molecular grade substitute.)

Equipment Checklist

Check to ensure that you have all of the necessary user-supplied equipment before proceeding to sample preparation.

- ▶ 4°C microcentrifuge (for ethanol precipitation)
- ▶ Benchtop microcentrifuge
- ▶ Dark Reader transilluminator (Clare Chemical Research, part # D195M) or UV transilluminator
- ▶ Electrophoresis power supply
- ▶ Room temperature tube rotator
- ▶ XCell Sure Lock Mini-Cell electrophoresis unit (Invitrogen, part # EI0001)

Dilute Oligos and MgCl_2 for v1.5 Protocol

This protocol ligates adapters to the 3' and 5' ends of the isolated small RNA.

Consumables

Illumina-Supplied

- ▶ 10X v1.5 sRNA 3' Adapter
- ▶ SRA RT Primer

User-Supplied

- ▶ Nuclease-free water
- ▶ 1 M MgCl_2 solution



NOTE

Prepare fresh dilutions of the adapter and primer with each use.

Procedure

1. Dilute the 10X v1.5 sRNA 3' adapter by mixing 1 μl adapter with 9 μl of nuclease-free water.
2. Dilute the SRA RT primer by mixing 1 μl primer with 4 μl nuclease-free water.
3. Dilute the 1 M MgCl_2 solution by mixing 100 μl with 900 μl nuclease-free water.

Ligate the v1.5 Small RNA 3' Adapter and 5' Adapter

This protocol describes the first ligation reaction of the v1.5 small RNA 3' adapter, followed by the 5' adapter ligation.

Consumables Illumina-Supplied

- ▶ Ultra Pure water
- ▶ RNaseOUT
- ▶ PR 5' adapter
- ▶ Diluted 1X v1.5 sRNA 3' adapter
- ▶ T4 RNA ligase



NOTE

The PR 3' adapter is not used in the v1.5 protocol. Use the v1.5 sRNA 3' adapter.

User-Supplied

- ▶ 100 mM MgCl₂
- ▶ T4 RNA Ligase 2, truncated (NEB-supplied)
- ▶ 10X T4 RNL2 truncated reaction buffer (NEB-supplied)
- ▶ 10 mM ATP

Procedure

1. Set up the ligation reactions in a sterile, nuclease-free 200 µl microcentrifuge tube using the following:
 - Total RNA in nuclease-free water (5.0 µl)
 - Diluted 1X v1.5 sRNA 3' adapter (1 µl)
2. Incubate at 70° C for 2 minutes, then transfer immediately to ice.
3. Add the following reagents and mix well:
 - 10X T4 RNL2 truncated Reaction buffer (1 µl)
 - 100 mM MgCl₂ (0.8 µl)
 - T4 RNA Ligase 2, truncated (1.5 µl)
 - RNaseOUT (0.5 µl)
4. Incubate at 22°C for 1 hour.
5. With 5 minutes remaining, prepare the 5' adapter for ligation by heating it at 70°C for 2 minutes, then transferring it to ice.

Procedure

1. Add the following reagents to the ligation mixture from step 4 and mix well:
 - 10 mM ATP (1 µl)
 - SRA 5' adapter (0.5 µl)
 - T4 RNA ligase (1 µl)
2. Incubate at 20°C for 1 hour. Store at 4°C.

Reverse Transcribe and Amplify the Small RNA Ligated with Adapters

Reverse transcription followed by PCR is used to create cDNA constructs based on the small RNA ligated with 3' and 5' adapters. This protocol selectively enriches those fragments that have adapter molecules on both ends. The PCR is performed with two primers that anneal to the ends of the adapters.

Consumables

Illumina-Supplied

- ▶ Ultra pure water
- ▶ SRA RT primer
- ▶ RNaseOUT
- ▶ 5X Phusion HF Buffer (Finnzymes Oy)
- ▶ Phusion Polymerase (Finnzymes Oy)
- ▶ Primer GX1
- ▶ Primer GX2
- ▶ 25 mM dNTP mix

User-Supplied

- ▶ 5' and 3' adapter-ligated RNA (4.0 µl)
- ▶ SuperScript II Reverse Transcriptase with 100 mM DTT and 5X first strand buffer (Invitrogen, part # 18064-014)

Procedure

Prepare Template

1. Combine the following in a sterile, nuclease-free, 200 µl microcentrifuge tube:
 - Purified 5' and 3' ligated RNA (4.0 µl)
 - Diluted SRA RT primer (1.0 µl)
 The total volume should be 5 µl.
2. Heat the mixture at 65°C in a thermal cycler for 10 minutes.
3. Place the tube on ice.

Dilute the 25 mM dNTP Mix

1. Premix the following reagents in a separate, sterile, nuclease-free, 200 µl PCR tube. Multiply each volume by the number of samples being prepared. Make 10% extra reagent if you are preparing multiple samples.
 - Ultra pure water (0.5 µl)
 - 25 mM dNTP mix (0.5 µl)
 The total volume should be 1 µl.
2. Label the tube "12.5 mM dNTP Mix."

Perform Reverse Transcription

1. Premix the following reagents in the order listed in a separate tube. Multiply each volume by the number of samples being prepared. Make 10% extra reagent if you are preparing multiple samples.
 - 5X first strand buffer (2 μ l)
 - 12.5 mM dNTP mix (0.5 μ l)
 - 100 mM DTT (1 μ l)
 - RNase OUT (0.5 μ l)
2. Add 4 μ l of the mix to the cooled tube containing the primer-annealed template material.
The total volume should now be 9 μ l (5 μ l of template preparation and 4 μ l of reverse transcription).
3. Heat the sample to 48°C in a thermal cycler for 3 minutes.
4. Add 1 μ l SuperScript II Reverse Transcriptase. The total volume should now be 10 μ l.
5. Incubate in a thermal cycler at 44°C for 1 hour.

Prepare the PCR Master Mix

- ▶ Premix the following reagents in the listed order in a separate tube. Multiply each volume by the number of samples being prepared. Make 10% extra reagent if you are preparing multiple samples.
 - Ultra pure water (27 μ l)
 - 5X Phusion HF buffer (10 μ l)
 - Primer GX1 (1.0 μ l)
 - Primer GX2 (1.0 μ l)
 - 25 mM dNTP mix (0.5 μ l)
 - Phusion DNA Polymerase (0.5 μ l)
 The total volume should be 40 μ l.

Perform PCR Amplification

1. Add 40 μ l of PCR master mix into a sterile, nuclease-free 200 μ l PCR tube.
2. Add 10 μ l of single strand reverse-transcribed cDNA.
3. Amplify the PCR in the thermal cycler using the following PCR protocol:
 - a. 30 seconds at 98°C
 - b. 9 to 12 cycles of:
 - 10 seconds at 98°C
 - 30 seconds at 60°C
 - 15 seconds at 72°C
 - c. 10 minutes at 72°C
 - d. Hold at 4°C

**NOTE**

Amplification conditions may vary based on RNA input amount, tissue type, and species. This protocol was optimized using 1 µg of total RNA from mouse and human brain. The number of PCR cycles can be adjusted if clear and distinct bands are not observed in the gel image.

Purify the Amplified cDNA Construct

This protocol gel purifies the amplified cDNA construct in preparation for loading on the Illumina Cluster Station.

Consumables

Illumina-Supplied

- ▶ Ultra pure water
- ▶ 25 bp ladder
- ▶ 10X gel elution buffer
- ▶ Spin-X cellulose acetate filter
- ▶ Glycogen
- ▶ 1X resuspension buffer

User-Supplied

- ▶ Amplified cDNA construct (50 µl)
- ▶ 6% Novex TBE PAGE gel, 1.0 mm, 10 well
- ▶ 5X Novex TBE buffer
- ▶ Ultra pure ethidium bromide
- ▶ 3 M NaOAc, pH 5.2
- ▶ -20°C 100% ethanol
- ▶ Room temperature 70% ethanol
- ▶ Clean scalpels
- ▶ 21-gauge needles
- ▶ 6X DNA loading dye

Procedure

It is important to follow this procedure exactly to ensure reproducibility. Illumina does not recommend purifying multiple samples on a single gel due to the risk of cross-contamination between libraries. If multiple samples are run on a single gel, keep at least 4 empty wells between samples.

Prepare the Gel Electrophoresis Reagents and Apparatus

1. Determine the volume of 1X TBE buffer needed. Dilute the 5X TBE buffer to 1X for use in electrophoresis.
2. Assemble the gel electrophoresis apparatus per the manufacturer's instructions.

Run the Gel Electrophoresis

1. Mix 1 μ l of 25 bp ladder with 1 μ l of 6X DNA loading dye.
2. Mix 50 μ l of amplified cDNA construct with 10 μ l of 6X DNA loading dye.
3. Load 2 μ l of mixed 25 bp ladder and loading dye in one well on the 6% PAGE gel.
4. Load two wells with 25 μ l each of mixed amplified cDNA construct and loading dye on the 6% PAGE gel.
5. Run the gel for 30–35 minutes at 200 V or until the front dye exits the gel.
6. Remove the gel from the apparatus.

Dilute the 10X Gel Elution Buffer

- ▶ Dilute the 10X gel elution buffer into a fresh tube.

Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent mix if you are preparing multiple samples.

- Ultra pure water (90 μ l)
- 10X gel elution buffer (10 μ l)

The total volume should be 100 μ l.

Recover the Purified Construct

1. Puncture the bottom of a sterile, nuclease-free, 0.5 ml microcentrifuge tube 4–5 times with a 21-gauge needle.
2. Place the 0.5 ml microcentrifuge tube into a sterile, round-bottom, nuclease-free, 2 ml microcentrifuge tube.
3. Pry apart the cassette and stain the gel with the ethidium bromide in a clean container for 2–3 minutes.
4. View the gel on a Dark Reader transilluminator or a UV transilluminator.
The 25 bp ladder consists of 18 dsDNA fragments between 25 bp and 450 bp in 25 bp increments plus a fragment at 500 bp. An additional fragment at 2652 bp is provided above the ladder. The 125 bp is approximately 2–3 times brighter than all bands except the 500 bp and 2652 bp bands to provide internal orientation.

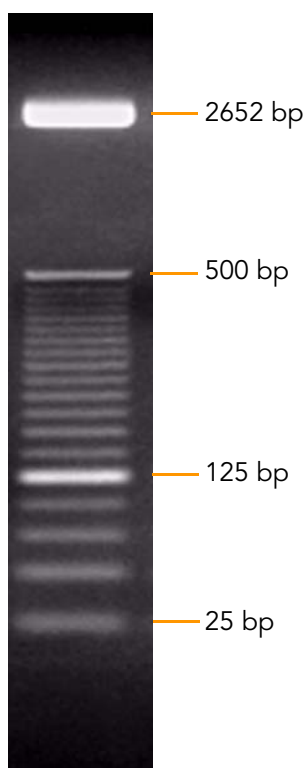


Figure 4 25 bp Ladder

5. Using a clean scalpel, cut out the bands corresponding to approximately the adapter-ligated constructs derived from the 22 nt and 30 nt small RNA fragments.

The band containing the 22 nt RNA fragment with both adapters will be a total of 93 nt in length. The band containing the 30 nt RNA fragment with both adapters will be 100 nt in length.

6. Place the band of interest into the 0.5 ml microcentrifuge tube from step 1.
7. Centrifuge the stacked tubes at 14000 rpm in a microcentrifuge for 2 minutes at room temperature to move the gel through the holes into the 2 ml tube.
8. Add 100 μ l of 1X gel elution buffer to the gel debris in the 2 ml tube.
9. Elute the DNA by rotating the tube gently at room temperature for 2 hours.
10. Transfer the eluate and the gel debris to the top of a Spin-X filter.
11. Centrifuge the filter for 2 minutes at 14000 rpm.
12. Add 1 μ l of glycogen, 10 μ l of 3M NaOAc, and 325 μ l of -20°C 100% ethanol.
13. Immediately centrifuge to 14000 rpm for 20 minutes in a benchtop microcentrifuge.
14. Remove and discard the supernatant, leaving the pellet intact.

15. Wash the pellet with 500 μ l of room temperature 70% ethanol.
16. Remove and discard the supernatant, leaving the pellet intact.
17. Dry the pellet using the speed vac.
18. Resuspend the pellet in 10 μ l resuspension buffer.

Select Small RNA Libraries

Below are two gel images representing small RNA libraries generated from human and mouse brain total RNA and a third library made from small RNA fragments purified from 1 μ g of human brain total RNA.

Sequencing can be conducted on individual bands or from pooled bands. The 93 nucleotide band primarily contains mature microRNA generated from approximately 22 nucleotide small RNA fragments.

A second band containing piwi-interacting RNAs, as well as some microRNAs and other regulatory small RNA molecules, corresponds to 100 nucleotides in length and is generated from approximately 30 nucleotide RNA fragments.

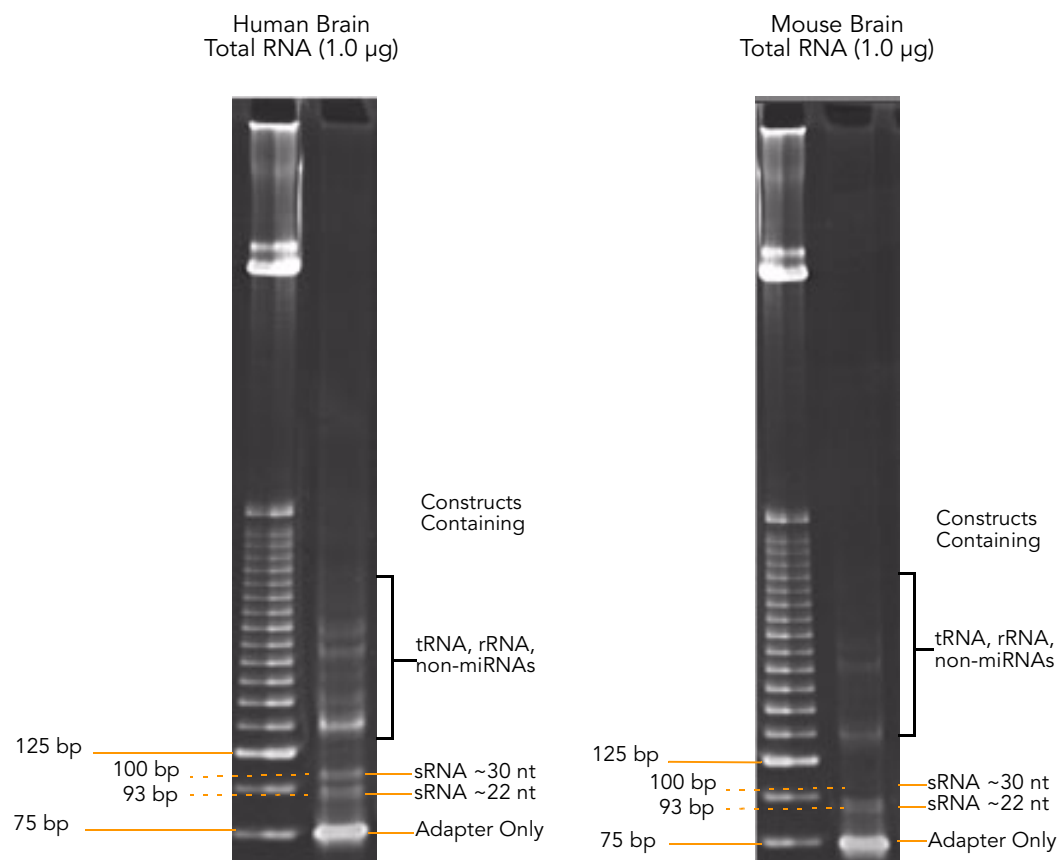


Figure 5 Small RNA Libraries from Total RNA

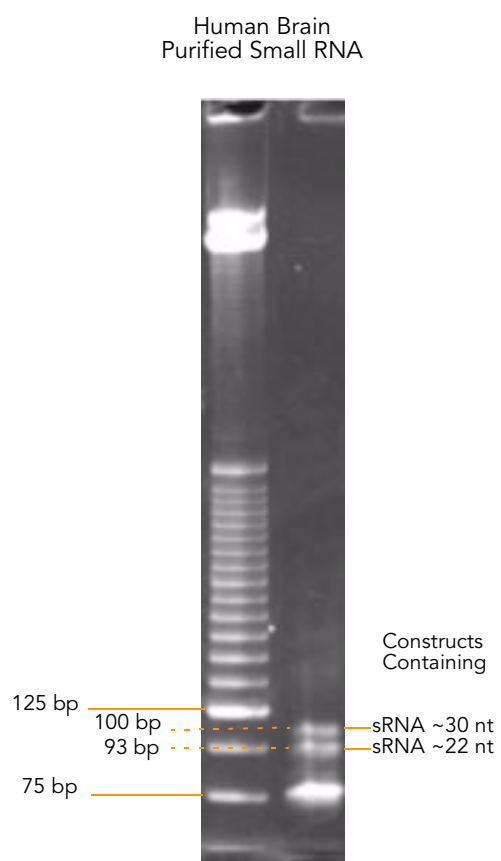


Figure 6 Purified Small RNA Libraries

Validate the Library

Illumina recommends performing the following quality control analysis on your sample library.

1. Load 1 μ l of the resuspended construct on an Agilent Technologies 2100 Bioanalyzer.
2. Check the size, purity, and concentration of the sample.
You can confirm the final product by cloning 1 μ l of the product into Invitrogen Zero Blunt TOPO vector, and sequence using conventional technology.

Illumina, Inc.
9885 Towne Centre Drive
San Diego, CA 92121-1975
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com

